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Light Microscopy of Periphrtal Nerve Adequately Fixed for Electron Microscopy

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LIGHT MICROSCOPY OF PERIPHERAL NERVE ADEQUATELY FIXED FOR ELECTRON MICROSCOPY

by

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This thesis submitted by Leonard L. Gunderson in partial fulfillment of the requirements for the Degree of Master of Science in the University of North Dakota is hereby approved by the Committee under whom the work has been done.

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ABSTRACT

Electron microscopy has settled prior controversies regarding the relationship of axon, myelin and Schwann cell but doubt remains about the correct interpretation of the classic binding sheaths. Perineurium is customarily interpreted to be flattened fibroblasts but recent evidence suggests attenuated Schwann cells. This study attempts to reinterpret the morphology of peripheral nerve known to be fixed adequately for fine structure. Special emphasis is placed on the binding sheaths, especially perineurium.

Forty white rats were perfused with buffered aldehydes. Sural and sciatic nerves were removed. Smaller nerves were obtained with orbital contents and the loose connective tissue of spleen and pancreas. These were prepared for light microscopy with the least possible change of routine techniques. Selective chromatic stains were modified to demonstrate nerve morphology.

In this study perineurium is distinguished from epineurium and endoneurium because its boundary membranes stain with the periodic acid-Schiff (PAS) method and counterstains. Additional stains also show perineurium as a distinct morphological layer that is complete and organized. Blood vessels are surrounded by perineurium in their longitudinal course within this sheath. All nerve branches acquire a perineural covering from a parent nerve by a process of gradual partitioning.

Perineurium is demonstrable as a specialized layer. It resembles Schwann cells by reason of its boundary membranes and "wrap-around" tendencies. This suggests neurectodermal rather than mesodermal origin. Tissue space with its contained connective tissues exists both inside and outside of perineurium.

CHAPTER I

INTRODUCTION

Peripheral nerve has been extensively studied by electron microscopists during the past fifteen years (1-7, 14-26). The patterns of fine structure thus revealed have settled prior controversies regarding the relationship of axon, myelin and Schwann cells, but serious doubt remains as to the correct interpretation of the binding sheaths of peripheral nerve. It seemed logical, therefore, that a new interpretation of peripheral nerve might be derived from tissue known to be fixed adequately for fine structure but otherwise prepared by methods standard for light microscopy.

Theodore Schwann's early studies laid the foundation for later interpretations and changes in the light microscopy of peripheral nerve (26). It should be noted that he assumed too close and analogy between nerve and muscle, which is especially evident in his description of nerve development. He stated that primary cells, at first indistinguishable from the surrounding cells, acquired identity as nerves when they became arranged in rows to form secondary cells. A layer of white matter (myelin) was then deposited which separated the cell membrane of the original secondary or Schwann cell from an inner core (or neuraxon) (26, 27). Nearly forty years later, Ranvier was the first to interpret the neuraxon correctly as a peripheral process of the nerve cell body (26, 28-30). It was early recognized that some neuraxons are enveloped in both a myelin sheath and

a sheath of Schwann whereas others possess only the sheath of Schwann. To Schwann, the unmyelinated (Remak) fibers were simply chains of secondary cells without myelin deposition which exhibited nuclei along their length (26). The general opinion of others was that in unmyelinated fibers neuraxons were surrounded by Schwann cell cytoplasm and differed from myelinated fibers by the absence of a myelin sheath between the neuraxon and the Schwann cell. Early workers could not agree as to the exact origin of the myelin sheath. Harrison in 1906 (31, 32) felt that myelin came from the Schwann cell. He had proved neural crest origin for these cells and noted that in their absence myelin sheaths would not develop. His explanation of the process was that sheath cells wrapped themselves around a fiber and became loaded with fat droplets. Others (31, 33, 34) felt that the axon was the inducer for subsequent myelin derivation from the intercellular substance between the fiber and the sheath cell. This concept, they felt, was supported by the fact that myelin broke down when the axon degenerated. A compromise opinion, based on regeneration studies (36), admitted that both axon and Schwann cell were necessary for proper myelin formation. Against this background of opinion, textbooks presented nerves as parallel aggregates of neuraxons with or without myelin sheaths but each enclosed in a sheath of Schwann (or neurilemma). These elements, all of which are of neurectodermal origin (31, 32) were generally believed to be bound together by connective tissue sheaths which were mesodermal in their origin. Epineurium was regarded as the connective tissue binding the entire gross nerve while each individual fiber possessed its own endoneurium. Perineurium existed as an intermediate sheath partitioning bundles of neuraxons within a gross nerve. These sheaths were directly comparable to the epimysium, endomysium and perimysium of skeletal muscle

(31, 37, 38).

Electron microscopy has done much to clarify prior controversies regarding peripheral nerve. Gasser (1952), ben Geren (1954) and Robertson (1955) have provided a unified concept of relationships among axon, myelin and Schwann cell. Gasser (18, 19) showed that neuraxons of unmyelinated fibers ran in gutters formed by plasmalemmal indentations and were not completely surrounded by Schwann cell cytoplasm as originally believed. He proved that the gap of 100-150°A, which remained between the nearly apposed lips of Schwann cell plasmalemma, was totally independent of the depth of axon embedment within the Schwann cell. The term mesaxon was coined by Gasser to describe the appearance of the suspension of the axon by Schwann cell plasmalemma in cross section. Betty ben Geren (20) worked with embryological material and observed fibers arranged like Gasser's unmyelinated fibers but with only one axon per Schwann cell. Certain of these had long mesaxons spiraled loosely around the axon in several turns. This led her to suspect that the myelin sheath might actually be a mesaxon that was greatly drawn out and wound around the axon in a tightly packed spiral. Robertson (21-23) confirmed Miss ben Geren's findings with electron micrographs of myelin sheaths which showed inner and outer mesaxon loops and a continuity with the plasmalemma of the Schwann cell. Although the above findings are now accepted by modern textbooks, the histology of the binding sheaths of peripheral nerve has not yet been rewritten (37-39). Electron microscopy has revealed typical formed elements of connective tissue in both epineurium and endoneurium (4-7). However, the cellular sleeves usually interpreted as perineurium bear closer morphological resemblance to attenuated Schwann cells than to the fibroblasts of connective tissue (1-4, 6, 7). This casts doubt on the interpretation of

these sheaths that was formulated in the early days of light microscopy (28, 35, 36, 40-42).

A new avenue of approach for re-interpretation of peripheral nerve at the light microscope level is afforded by certain recently developed refinements of fixation for electron microscopy. It has long been known that the ordinary fixation methods of light microscopy are inadequate for the preservation of fine structure (43, 44). Buffered fixation (44, 45) overcame this, and for more than a decade, osmium tetroxide was the reagent of choice. However, this technique had limited application outside of electron microscopy since tissue blocks had to be very small and subsequent chromatic staining was seriously inhibited. Refinement of fixation by perfusion (46) later provided well-fixed tissues in bulk. This was soon followed by perfusion with buffered aldehyde fixatives (47-52) which yielded tissues adequately fixed for fine structure yet amenable to chromatic staining.

Thus, there seemed to be no reason why these methods could not be applied to light microscopy with profit. Problems incident to this transfer were worked out in collaboration with Mr. William C. Rosen and Charles R. Basom during the summer of 1965 (53). This thesis accordingly reports a histological analysis of peripheral nerve known to be fixed for fine structure and prepared thereafter for light microscopy with the least possible alteration of standard techniques.

CHAPTER II

METHODOLOGY

The techniques utilized for this study closely followed the approach worked out in the summer of 1965 in collaboration with Mr. William C. Rosen and Mr. Charles R. Basom. A detailed description of this work is found in Appendix II (pp. 41-50). It offers a standardized method for obtaining general body tissues well-fixed for fine structure and for preparing these tissues for light microscopy with the least possible change of routine techniques.

Forty ~~animals~~ white rats were perfused (46) with buffered aldehydes (47-49) and nervous tissue was obtained from twenty-eight of these. The entire animal usually perfused readily but arterial clamps were occasionally used on the innominate arteries to insure good p~~e~~reservation of sciatic and sural nerves. These were removed for study as examples of larger nerves. Smaller nerves associated with the orbit, spleen and pancreas were also obtained. After removal, a few of these tissues were post-fixed in osmic acid (46). Peripheral nervous tissue presented special problems not encountered with other, less demanding tissues. These complications are described in some detail below.

The extreme brittleness of nervous tissue necessitated special precautions during its removal. Large peripheral nerves and orbital contents presented separate problems. The sural and sciatic nerves suffered the least amount of damage if removed with adjacent muscle. In the rat the

main part of the sciatic nerve runs directly beneath the divisions of the biceps femoris muscle after entering the thigh at the sciatic notch (54). Therefore, the heads of the biceps femoris were separated by forceps dissection to locate the nerve so that the overlying muscle could be carefully removed. Distortion (bending or stretching) was prevented by cutting the connective tissue which bound the nerve to the muscle with a sharp scalpel or razor blade. Portions of the nerve together with underlying muscle were then removed. The sural nerve was located most easily in its superficial position on the lower posterior portion of the gastrocnemius muscle (54). Tissues were removed in this area and were also removed further cephalad with the surrounding gastrocnemius and biceps muscles. The orbital contents were removed by either of two approaches. When preservation of the brain was desirable, removal was by anterior dissection. The periosteum was peeled away from the bone and the remainder of the orbital contents separated from the temporalis muscle. It was then possible to cut the soft tissues near the origins of the extra-ocular muscles and to remove the orbital contents in one piece. The alternate procedure was to remove the medial bony wall of the orbit with scissors and forceps followed by removal of the soft parts as described above. Although both methods could yield undamaged preparations, utilization of the latter procedure, though more time consuming, assured better tissue (54).

Dehydration, clearing, embedment and microtomy followed the procedures described in Appendix II (pp. 43, 44), but it was necessary to exercise all of the precautions described. There was noticeable difficulty with spreading during mounting, a characteristic of peripheral nerve. This could not be overcome as with most other tissues merely by using double embedment procedures. It was necessary to use a minimum amount of water

on the slide and to leave slides on the hot plate only until the water had evaporated. Slides were then "flamed" to insure adherance of the tissue to the albuminized slide.

A wide range of stains proved useful in analyzing peripheral nerve. These included: Hematoxylin (Harris's) and eosin, Snook's reticulin and Masson's trichrome, the characteristics of which are described more fully in Appendix II (p. 45). Further useful stains included periodic acid-Schiff (PAS), PAS and counterstains and Holmes' silver stain. The PAS and PAS and counterstain procedures followed the McManus method for PAS and hematoxylin (55) as found in Preece (56) with basic variations (57-59). Times in the Schiff reagent were decreased, blocking agents were used (Appendix II, p. 46), all tap water baths were replaced by distilled water, and the hematoxylin counterstain was used, left out or substituted for by acetate buffered cresyl violet (60). The Holmes' silver technique for paraffin sections (61-63) differed from most other stains in that standard times yielded the best differentiation. This stain, though somewhat erratic, produced an excellent picture of peripheral nerve.

CHAPTER III

OBSERVATIONS

The observations made in this thesis are based on tissues whose fixation differs from that for standard light microscopy. Early electron microscopy showed that conventional light microscopic fixation was inadequate for fine structure. Figures 1 and 2 illustrate the same finding with light microscopy. Nerve fixed by immersion in unbuffered 10% formalin (fig. 1) shows globular artifacts which mask the accepted axon-myelin picture. However, fixation of nerve with buffered aldehyde perfusion does preserve accepted structural relationships (fig. 2).

The general appearance of most nerves observed in this study is one of compactness. This is revealed by various stains in all sizes of nerves and in nerves taken from different areas of the rat. Sciatic nerve (fig. 3), medium-sized (fig. 4) and small-sized nerves (fig. 5) of the orbit indicate no change in degree of compactness. Small nerves associated with the pancreas (fig. 25) and the spleen (fig. 26) also exhibit this characteristic.

The relationships of axon, myelin and Schwann cell are not emphasized in this study. Nevertheless, some interesting things can be noted about each of them. Interesting comparative observations are made on tissues fixed by aldehyde perfusion and exhibiting varying degrees of post-fixation with osmic acid. In sciatic nerve which was not post-osmicated (fig. 6), a normal compactness of nerve and relation of epineurium to

perineurium exist but distortion in the form of axon displacement occurs. When present this is found only in larger nerves. Complete post-osmication of a small orbital nerve (fig. 8) produces an accepted picture of myelin throughout. Partial post-fixation of a larger nerve of the orbit (fig. 7) provides an interesting contrast. Complete preservation of the myelin is visible in only the lower portion of the nerve. In the upper half complete absence of myelin with subsequent axon displacement is noted as is the cartwheel pattern of neurokeratin (remains of dissolved myelin). Other figures of sciatic nerve fixed without post-osmication (figs. 9, 11, 12) show axons which are centrally placed. This condition is far more prevalent than that of displaced axons even in the large nerves. These figures also indicate interesting Schwann cell-axonal relationships. The "wrap-around" appearance of Schwann cell nuclei and cytoplasm in relation to both small and large, single myelinated axons shows clearly (figs. 9, 11). There is no indentation of the myelin by these nuclei. In figure 12 at least one nucleus is seen in relation to unmyelinated axons. These axons are somewhat smaller than the smallest myelinated fibers noted in the same field.

The binding sheaths of peripheral nerve; epineurium, endoneurium, and perineurium, are customarily thought to be formed of connective tissue. These sheaths are the focal point of this study since it is here that fixation for fine structure reveals the most significant departure from the accepted pattern of histological organization in peripheral nerve.

Endoneurium is displayed to good advantage by three different stains: Snook's reticulin, Masson's trichrome, and hematoxylin and eosin. Typical reticular fibers are demonstrated in the tissue space of endoneurium (fig. 10) by the reticulin stain, and lightly-stained collagen bundles are shown

by hematoxylin and eosin directly beneath the perineurium (figs. 21-24). Endoneural fibroblasts are most easily distinguished by their shape in a position just inside the perineurium. They differ from perineural nuclei both by location and by absence of a boundary membrane surrounding the cell (figs. 13, 14). Mast cells with heavy granules are present in certain preparations.

While endoneural composition and appearance are the same in nerves from all areas of the body, the regional organization of epineurium differs noticeably. This is most evident when comparing the sciatic nerve to nerves of the orbit. The epineurium of the sciatic nerve is composed of bundles of dense collagen next to the perineurium and becomes more loosely arranged in a peripheral position. A small number of fibroblasts intermingle with these bundles (figs. 3, 21-24). In contrast the epineurium of most orbital nerves (figs. 2, 4, 5, 15-20) is poorly organized and is present as a loose arrangement of collagen fibers and fibroblasts. Mast cells are occasionally seen in the epineurium.

Perineurium can be distinguished most clearly from the other binding sheaths in preparations stained with PAS and counterstains (figs. 2, 4, 5, 15-20). The boundary (basement) membranes (67, 68) of the perineurium stain positively and thus define perineural limits. Using these findings it is possible to reinterpret other stains and recognize a distinctive perineurium. Holmes' silver stain (fig. 3), Masson's trichrome (fig. 6), and hematoxylin and eosin (figs. 21-24) all provide this distinction but the first two stains are somewhat limited. They leave the perineurium so dark that nuclei are not easily recognized, whereas hematoxylin and eosin has stained the perineurium denser than the epineurium yet leaves visible nuclei. Besides establishing this distinction, the above techniques re-

vealed perineural layering, perineural partitioning and the perineural relation to blood vessels.

The PAS stain and to a lesser degree hematoxylin and eosin are most useful when studying the details of perineural layering. While it is not always possible to count the cellular layers of perineurium with accuracy in the light microscope, it is generally noticable that smaller nerves possess fewer layers than do larger ones. At least two layers are clearly indicated in several figures of larger orbital nerves (figs. 4, 13, 14). The nuclei of the two layers lie nearly one upon the other and the thin cytoplasmic layer, characteristic of perineurium, extends away from each nucleus. In a small orbital nerve (fig. 5) both the endoneural and epineural boundary membranes of a single-layered perineurium can be distinguished. As an additional example, the sciatic nerve (figs. 21-24) possesses a perineural sheath that is obviously thicker and has more layers than the perineurium of the small nerves of loose connective tissue (figs. 25, 26).

Perineurium anticipates nerve branching. It surrounds entire nerves as an unbroken sleeve in preparations where nerve branching is not imminent. As the site of branching is approached, nearby sections of tissue show evidence of perineural partitioning (figs. 15-20). These partitions, at first incomplete, gradually divide the nerve into its peripheral branches which eventually possess individual perineural sheaths.

Blood vessels pass into the endoneurium by traveling longtudinally within the perineurium for some distance (figs. 21-24). During this stage, at least one layer of perineurium passes around each side of the vessel.

CHAPTER IV

DISCUSSION

The observations made in this study substantiate prior findings on axon, myelin and Schwann cell relationships up to the limits imposed by light microscopy. The works of Gasser (18, 19), ben Geren (20) and Robertson (21-23) are essentially supported by this work. The findings of electron and light microscopy closely complement each other so as to leave little doubt as to the reliability of the evidence.

The problem of myelin fixation became a point of interest when axon displacement was noticed in certain preparations. This distortion is thought to be caused by dissolution of myelin traceable to dehydration and clearing procedures following aldehyde fixation. This type of fixation preserves the myelin but does not render it immune to subsequent action by organic solvents such as alcohol, xylene and chloroform. This is avoided in electron microscopy by post-osmication prior to the use of organic solvents. Post-osmication also yields good myelin preservation for light microscopy but its advantage is counteracted because the response of the tissues to chromatic stains is severely inhibited. Both situations are illustrated in a single field in figure 7. In this preparation only part of the nerve is fully osmicated. The fully-osmicated portion shows excellent preservation of the tissue but the hematoxylin stain is weak. Where osmication does not blacken the tissue the chromatic dye stains with high contrast, but the myelin is poorly preserved. Fortunately, in this

study it was not necessary to post-osmicate tissues routinely since axon displacement did not occur in small nerves (figs. 4, 5, 25, 26) and was observed only occasionally in the larger ones. Subtleties of fixation may have been responsible, since myelin fixation in large nerves which showed axon displacement was often unusually good in areas close to neural capillaries.

The binding sheaths of peripheral nerve, classically known as epineurium, endoneurium and perineurium, merit close analysis in the special preparations used in this study. All three of these sheaths are customarily interpreted as belonging to the connective tissues (28, 35, 36, 40-42). Epineurium and endoneurium fit into this category well. However, there is good reason to doubt the identity of perineurium as a member of the connective tissue family.

Epineurium has no characteristics that distinguish it from ordinary connective tissue. Its degree of organization as a sheath enclosing peripheral nerve varies markedly from one location to another. This is illustrated by its structural coherence around the sciatic nerve and by the lack of any evident binding characteristics around orbital nerves. It may be interpreted to be the connective tissue lying just external to the perineurium.

Endoneurium, although spatially restricted by the enclosing perineurium and the longitudinally running nerve fibers, is unmistakably composed of connective tissue. Reticular fibers are clearly demonstrable within it (fig. 10) and occasional collagen fibers and fibroblasts can be observed near the inner border of the perineurium (figs. 21-24, 13, 14). Occasional mast cells occur here, as well as in the epineurium.

Electron microscopy supports this interpretation (4-7, 76) since typical formed elements of the connective tissues are found in both loca-

tions. Although there is some difference of opinion concerning populations of cell types (4, 6), all workers agree that both endoneurium and epineurium are composed of connective tissue.

Perineurium deserves special attention since both light microscopy and electron microscopy have shown that it differs markedly from connective tissue. Boundary membranes on both the endoneural and epineural sides of the perineurium stain positively with PAS and counterstains (55-60, 64, 65) and thus define perineural limits (figs. 4, 5, 15-20). The Holmes' silver stain, Masson's trichrome and hematoxylin and eosin also demonstrate distinct boundaries between perineurium and the two connective tissue binding sheaths (figs. 3, 6, 21-24). This conflicts with early light microscopic observations, which did not define specific perineural limits. Electron microscopists now use the presence of boundary membranes around perineurium to show that it differs from connective tissue (1-7). Burkel (2) has further shown that typical formed connective tissue cells do not form tight junctional complexes as seen in perineurium. He uses this fact, together with the presence of boundary membranes, to interpret perineurium as a non-connective tissue sheath.

Perineurium, as interpreted by the boundary membrane concept formulated by Low (66, 67), cannot be classified as a connective tissue. This concept holds that a constant pattern exists throughout the body whereby boundary membranes separate epithelium, nerve, muscle and fat from the tissue space, which contains connective tissue cells and extra-cellular fibers without boundary membranes (68).

Certain features of perineurium enable one to characterize it as resembling attenuated Schwann cells rather than flattened fibroblasts. The Schwann cells that surround axons possess the same boundary membrane as

does the perineurium, and both types of cells have the tendency to wrap-around structures traveling in a longitudinal direction. It is well accepted that the Schwann cell nucleus and cytoplasm wrap-around both myelinated and unmyelinated axons (18-23, figs. 9, 11, 12), and electron microscopic evidence has also shown that both Schwann cells and perineurium have this relation to longitudinally arranged reticular fibers (2, 26, 77). Blood vessels pass into the endoneurium by traveling longitudinally within the perineurium for some distance. At least one layer of perineurium has been seen to pass around each side of the vessel with both light (figs. 21-24) and electron microscopy (2). These observations further indicate the close resemblance of perineurium to Schwann cells in surrounding or engulfing longitudinally arranged structures.

Perineurium can exist as a single sleeve or as a sheath of few to many layers. The PAS stain and to a lesser degree hematoxylin and eosin have proven useful in analyzing the characteristics of these layers. Electron microscope findings have shown that certain of these layers are only 0.1 micron in width (2). Thus it is not always possible to determine the exact number of perineural layers with the light microscope. It is, however, generally noted and accepted that smaller nerves possess fewer layers than do large ones. Comparisons of sciatic (figs. 21-24) and orbital nerves (figs. 4, 5) illustrate this best at the light microscopic level and electron microscopic evidence supports this interpretation (1-7, 14).

The perineural coverings for nerve branches do not exist within the endoneurium as perineural partitions at all levels, as indicated by most textbooks (37-39). Instead, the perineurium can be seen in places as a structure which extends only around the outside of a nerve bundle. It may

anticipate nerve branching that will occur more peripherally by beginning the formation of septal partitions. These partitions, at first incomplete, eventually divide the nerve into its lower branches (figs. 15-20). Burkel (2) has shown at the electron microscopic level that the partitions always come from the inner perineural sleeve. Since the perineural coverings of nerve branches come from the parent nerve, this partially indicates why perineural layers are fewer in smaller nerves. Burkel has also illustrated that the perineural investment of small orbital nerves ends shortly before the nerve terminates at a motor end plate (1, 2). The exact order of termination is given as perineurium, myelin and finally nerve. This open-ended perineural sleeve provides continuity between the tissue space of endoneurium and epineurium and makes reasonable the finding of typical formed elements of connective tissue within each.

For some time peripheral nerve has been known to possess a functional diffusion barrier (69-74). This function was not fully understood when perineurium was still generally interpreted to be connective tissue. A more satisfying answer becomes available if perineurium is recognized as a complete, organized layer which has a boundary membrane on each side (fig. 4, 5, 15-20). Luft has shown that ruthenium red fails to penetrate the thin perineural layers unless damage is present (75).

Shanthaveerappa and Bourne have made an extensive study of perineurium (8-14). While they have undoubtedly observed the layer in question, their basic interpretation differs from the general trend. They distinguish between the cell types of peripheral nerve by means of chromatin patterns which are not seen in tissues prepared by aldehyde perfusion. They further recognize both a perineural epithelium and perineural connective tissue. No specific limits between perineurium and epineurium are provided

by this classification. This laboratory defines the perineural epithelium of these workers as a perineural sheath (single layer, perineural sleeve) and interprets any connective tissue outside of the perineurium as part of the epineurium. All connective tissue inside the perineurium is endoneurium.

To summarize, perineurium can be recognized and described at the level of light microscopy as a distinct morphological layer. By reason of its boundary membranes it is distinguished from epineurium and endoneurium and does not belong to the connective tissue family. Further distinctions such as its wrap-around tendency seems to align perineurium with Schwann cells and suggests a neurectodermal rather than a mesodermal origin. Perineural sheaths exist as sleeves which seem to anticipate nerve branching. All nerve branches acquire a perineural covering from the parent nerve by a process of perineural partitioning. Perineural sleeves are open ended. This allows a continuity of tissue space between epineurium and presents the major point at which the diffusion barrier of perineurium is not effective.

LEGEND

B	Nerve branch
EP	Epineurium
F	Fibroblast
I	Endoneural (inner) boundary membrane
M	Main nerves
O	Epineural (outer) boundary membrane
P	Perineurium
PN	Perineural nucleus
PP	Perineural partition
SN	Schwann cell nucleus

PLATE I

Fixation of nerve; perfusion versus immersion

Figure 1. Fixation by immersion in unbuffered 10% formalin produces globular artifacts in this longitudinal view of nerve. PAS and cresyl violet. 600X.

Figure 2. True structural relationships are maintained in nerves fixed by perfusion with buffered aldehydes. The perineurium (P) is easily distinguished from epineurium (EP) and perineural nuclei (PN) from fibroblasts (F). PAS and cresyl violet. 600X.

PLATE I

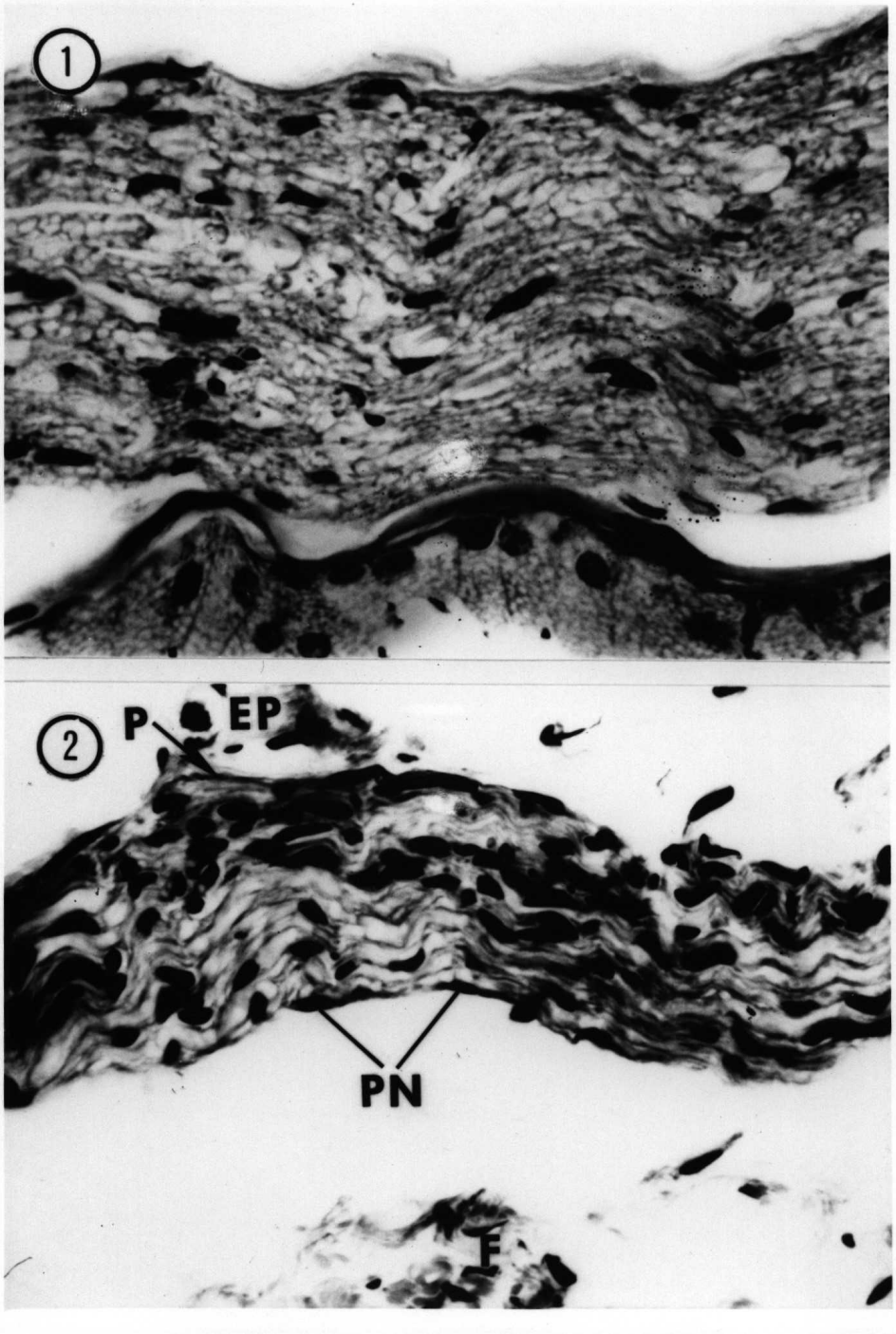


PLATE II

Figure 3. Compact large nerve. This entire sciatic nerve is very compact. The perineurium (P) is recognized as a dark band and the epineurium (EP) as lighter bundles with intermingled fibroblasts. Patent blood vessels are visible within the nerve (arrows). Holmes' silver. 200X.

PLATE II

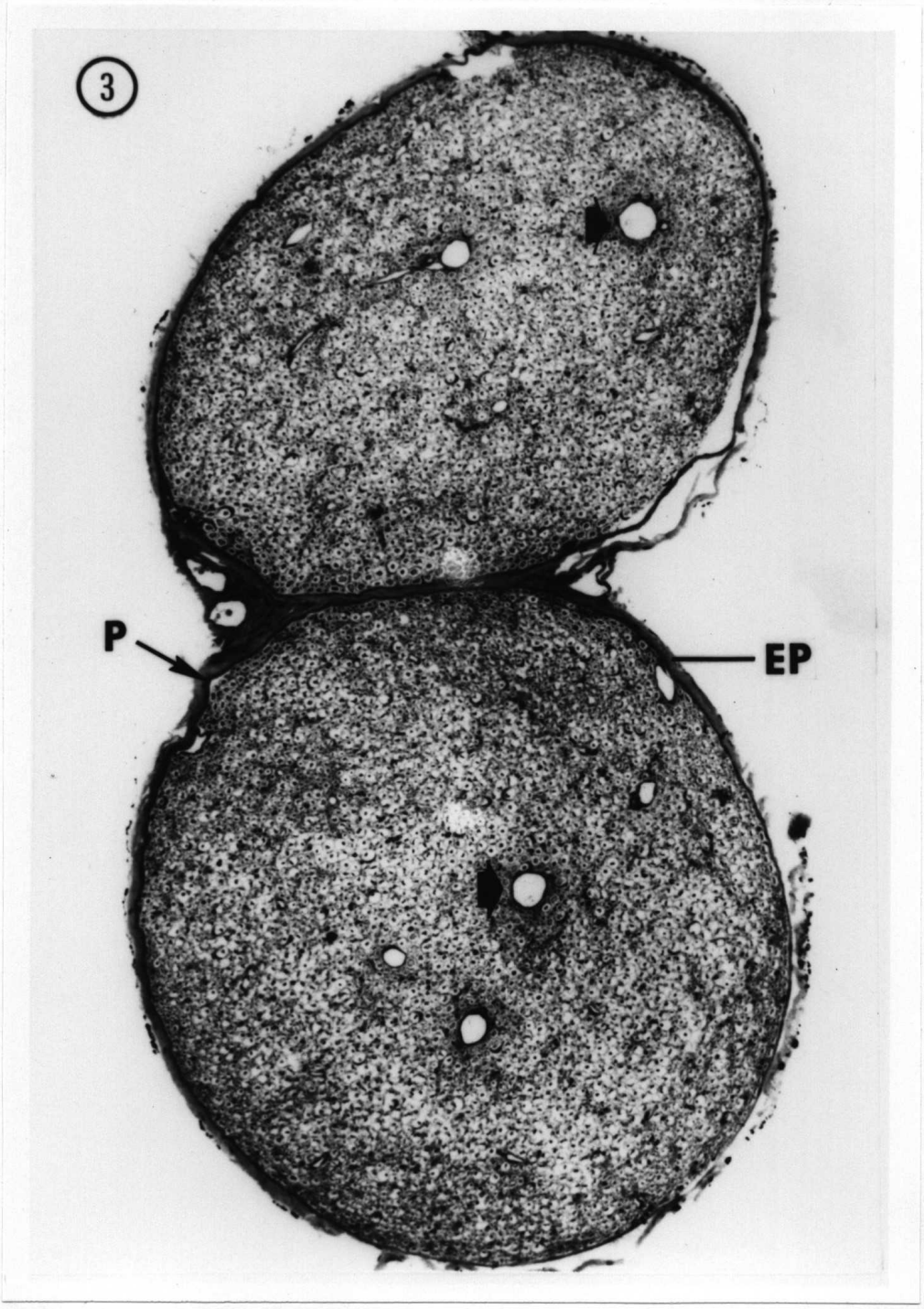


PLATE III

Figure 4. Double-layered perineurium; compact middle-sized nerve. This middle-sized nerve is very compact and shows evidence of a double-layered perineurium at the upper left (arrow). The nucleus (PN) of each layer is clearly seen. PAS and cresyl violet. 1600X.

Figure 5. Single-layered perineurium; compact small nerve. The small nerve illustrates PAS positive staining of both the endoneural (I) and epineural (O) boundary (basement) membranes of a single-layered perineurium. The perineural nucleus is easily distinguished from neighboring fibroblast nuclei (F). The compactness of this nerve is clearly seen. PAS and cresyl violet. 1600X.

PLATE III

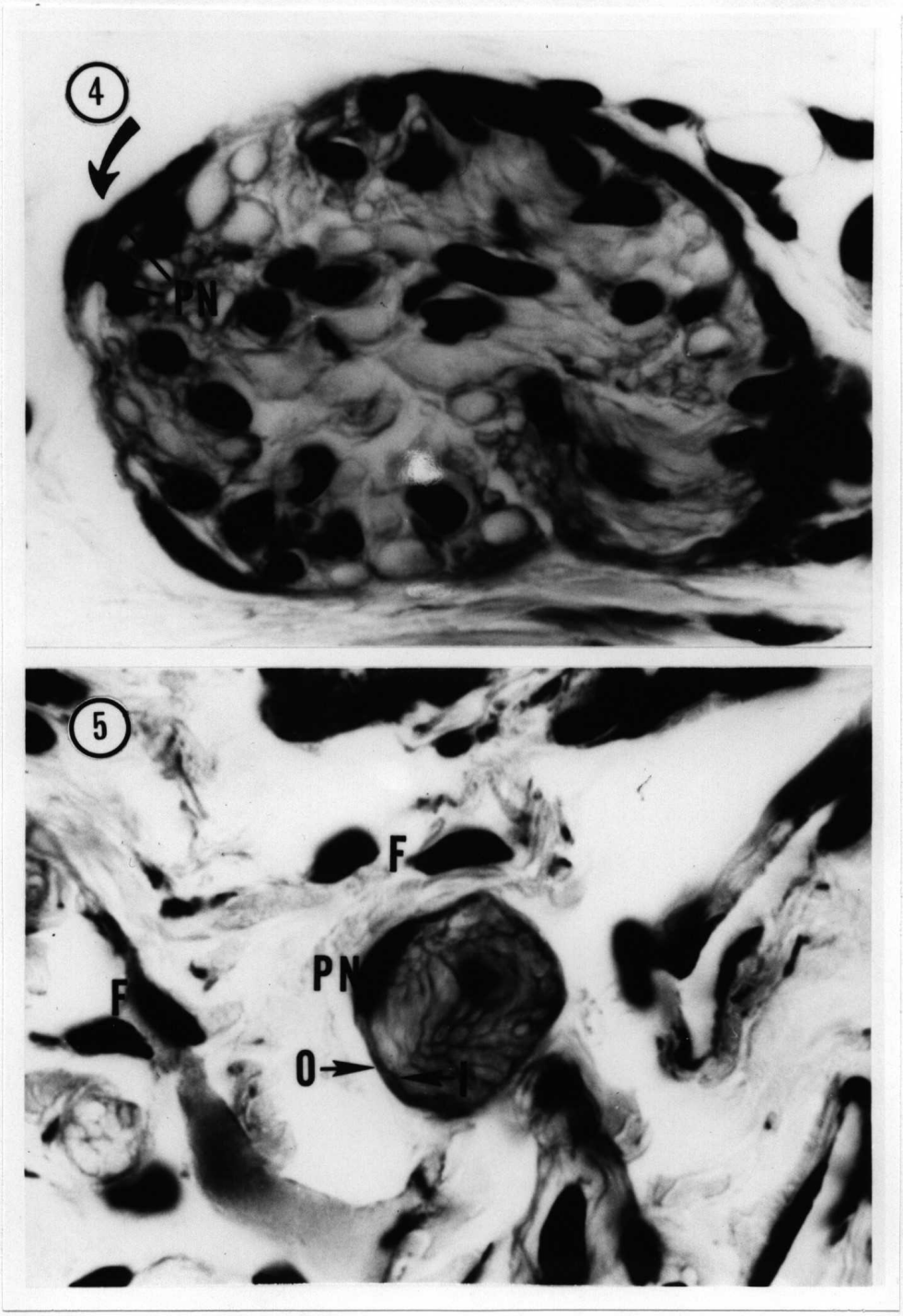


PLATE IV

Figure 6. No post-fixation with osmic acid. Much axon displacement is seen due to the dissolving of myelin by procedures following aldehyde perfusion. Epineurium (EP)-perineurium (P) relationships are well preserved. Masson's trichrome. 500X.

Figure 7. Partial post-osmication. Complete myelin preservation is evidenced in only the lower portion of this large nerve. Axon displacement (arrow) and varying degrees of myelin preservation are noted in the upper half. Schwann cell nuclei (SN) are easily seen in the poorly osmicated portion of the nerve since the stain is not inhibited as it is in the fully blackened portion. Hematoxylin (Harris's) and eosin. 600X.

Figure 8. Complete post-fixation. A true myelin picture shows in this entire smaller nerve. Schwann cell nuclei are stained but are difficult to see. Hematoxylin (Harris's) and eosin. 600X.

PLATE IV

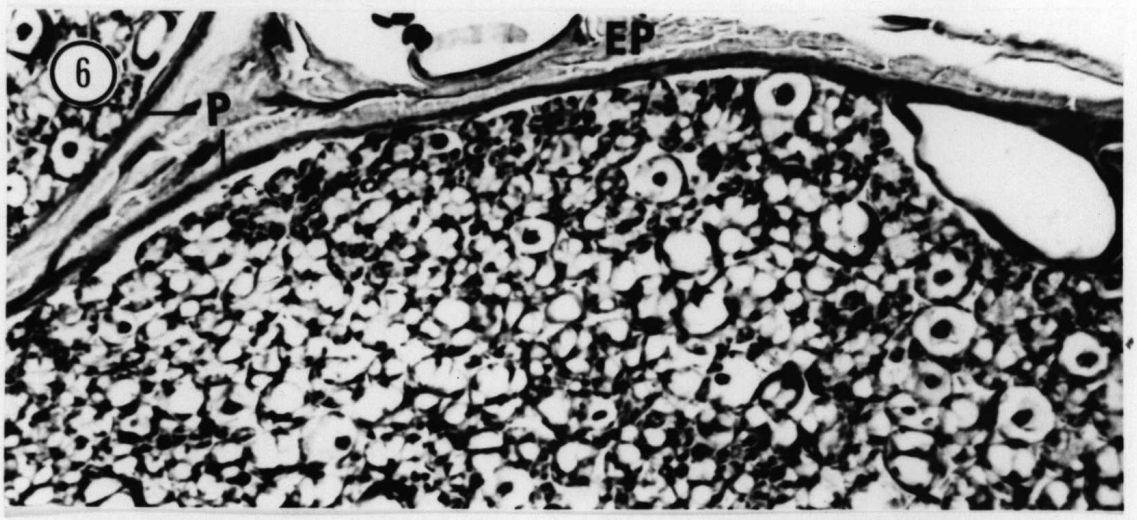


PLATE V

Figures 9 and 10. Nerve structure with selected stains. Peripheral nerve is shown to be very compact by both stains. In figure 9, centrally situated axons (arrow) are in relation to myelin and Schwann cells. Figure 10 shows typical reticular fibers in the tissue space of endoneurium. Holmes' silver and Snook's reticulin. 1300X.

PLATE V

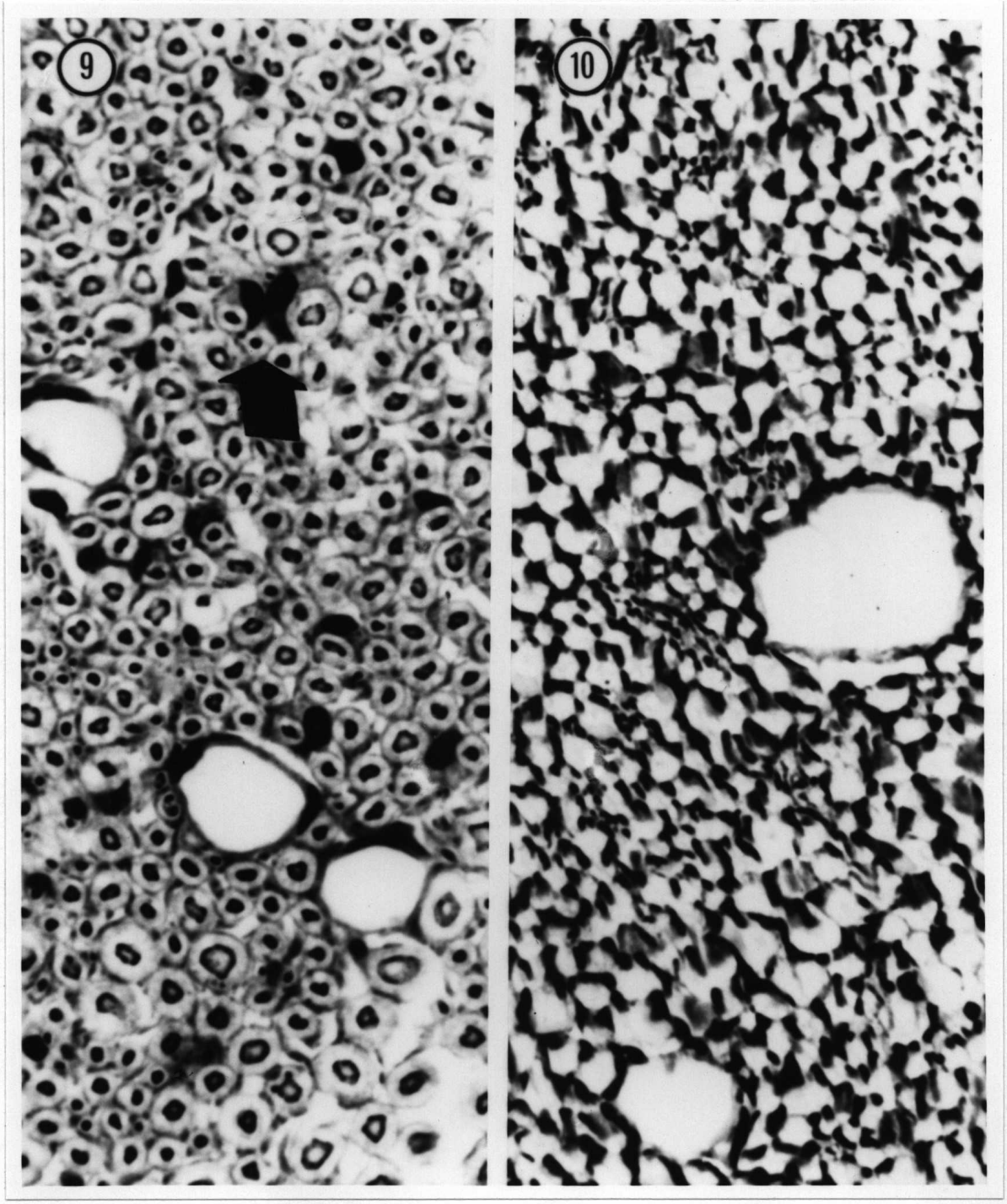


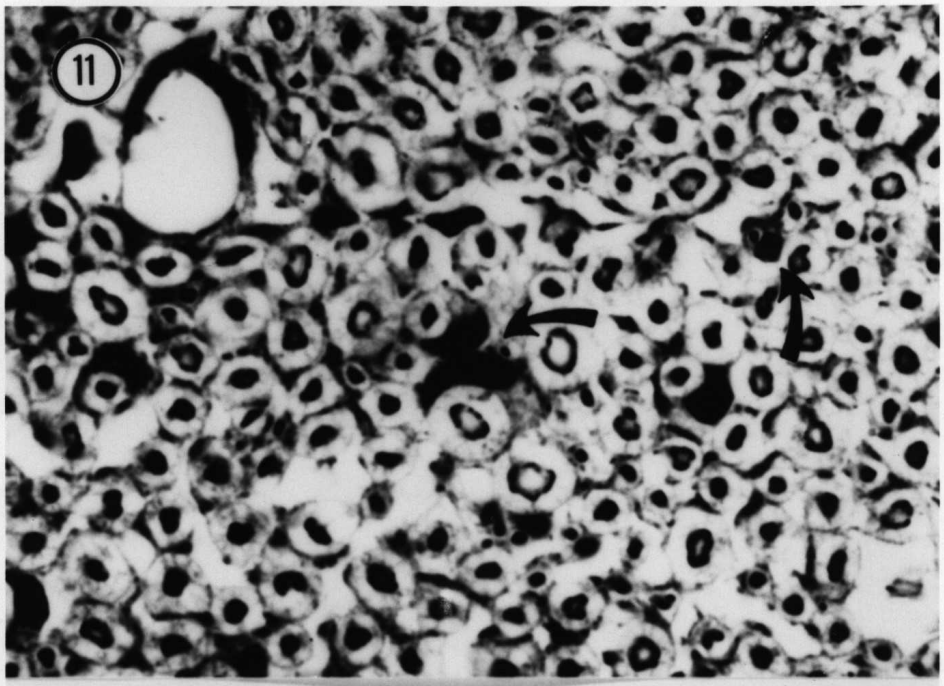
PLATE VI

Schwann cell relations to myelinated and unmyelinated axons

Figure 11. (Detail of figure 9) The center of this field shows nicely the wrap-around appearance of the Schwann cell nucleus and cytoplasm in relation to the myelin. Schwann nuclei are seen with both large and small myelinated axons (arrows). Holmes' silver. 1600X.

Figure 12. Unmyelinated axons can be seen (center arrow) sharing a single Schwann nucleus. Two small myelinated fibers with accompanying nuclei are seen at the right (arrow). Holmes' silver. 1600X.

PLATE VI



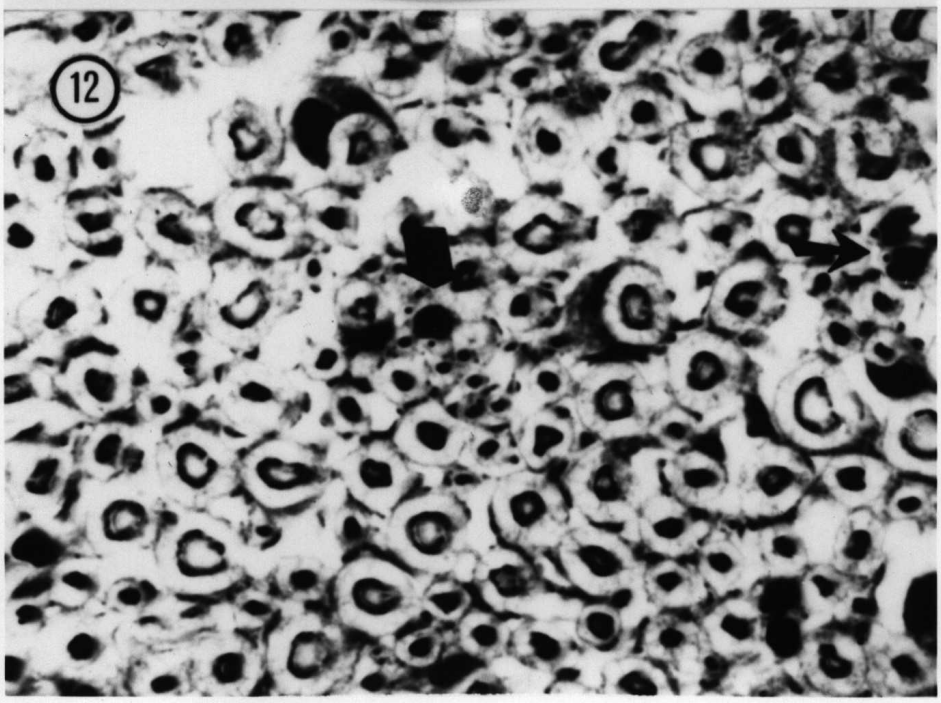
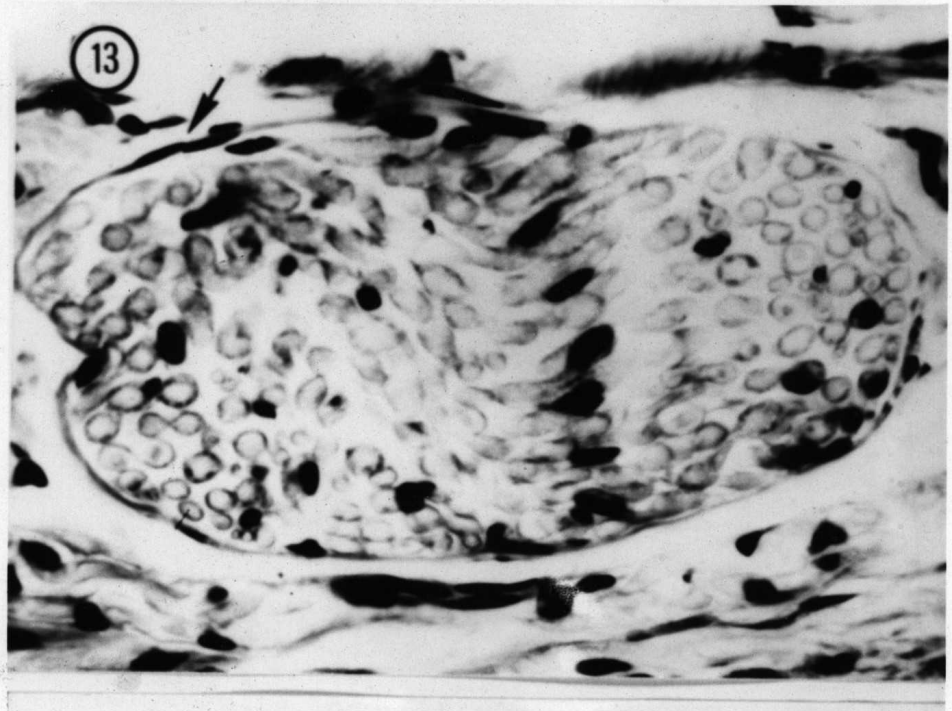


PLATE VII

Figures 13 and 14. Double-layered perineurium; endoneural fibroblast. Figure 14 (detail of figure 13) shows well a double-layered perineurium (arrow). An endoneural fibroblast (F) is seen directly below the nucleus (PN) of the inner perineural layer. PAS and cresyl violet. 600X, 1600X.

PLATE VII



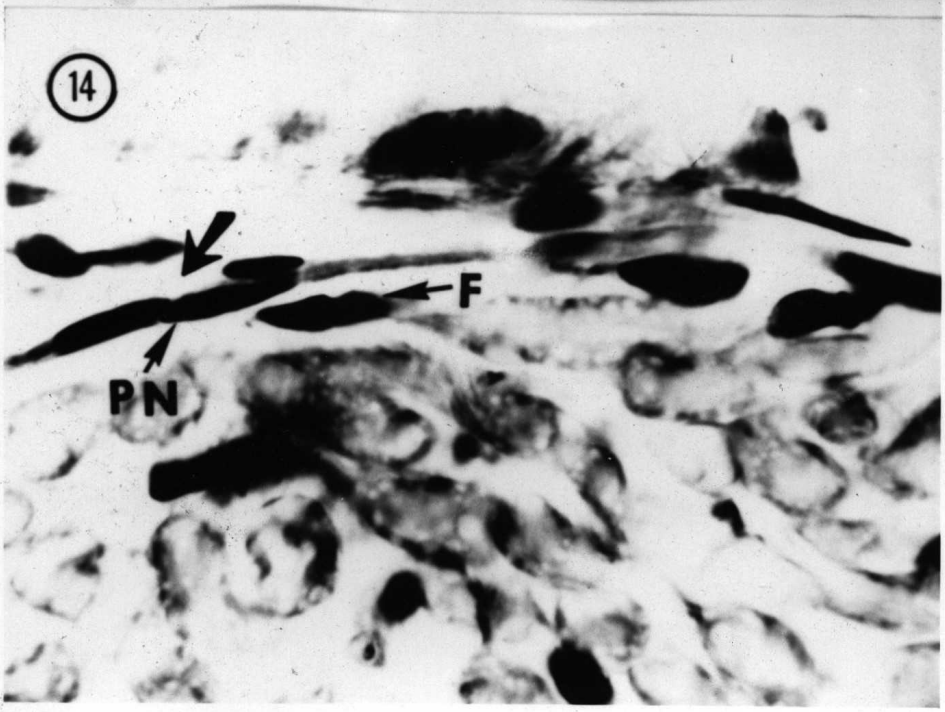


PLATE VIII

Perineurium anticipates nerve branching. Figures 15-18 show the acquisition of an individual perineurium by a nerve branch in stages. The perineurium is easily distinguished in all figures.

Figure 15. The large nerve shows the beginning of a perineural partition (PP). The smaller nerve has only the outer perineurium (P) with no partitions.

Figure 16. The perineural partition is now more distinct but is still incomplete. An incomplete partition has begun in the smaller nerve.

Figure 17. Partitions are now complete in both nerves. The upper nerve is distinctly divided into two parts, main portion (M) and branch (B), but the partition is possibly still shared by both.

Figure 18. The two portions of the upper nerve have separated. The lower nerve now has one complete and two incomplete partitions.

All figures are stained with PAS and cresyl violet. 300X.

PLATE VIII

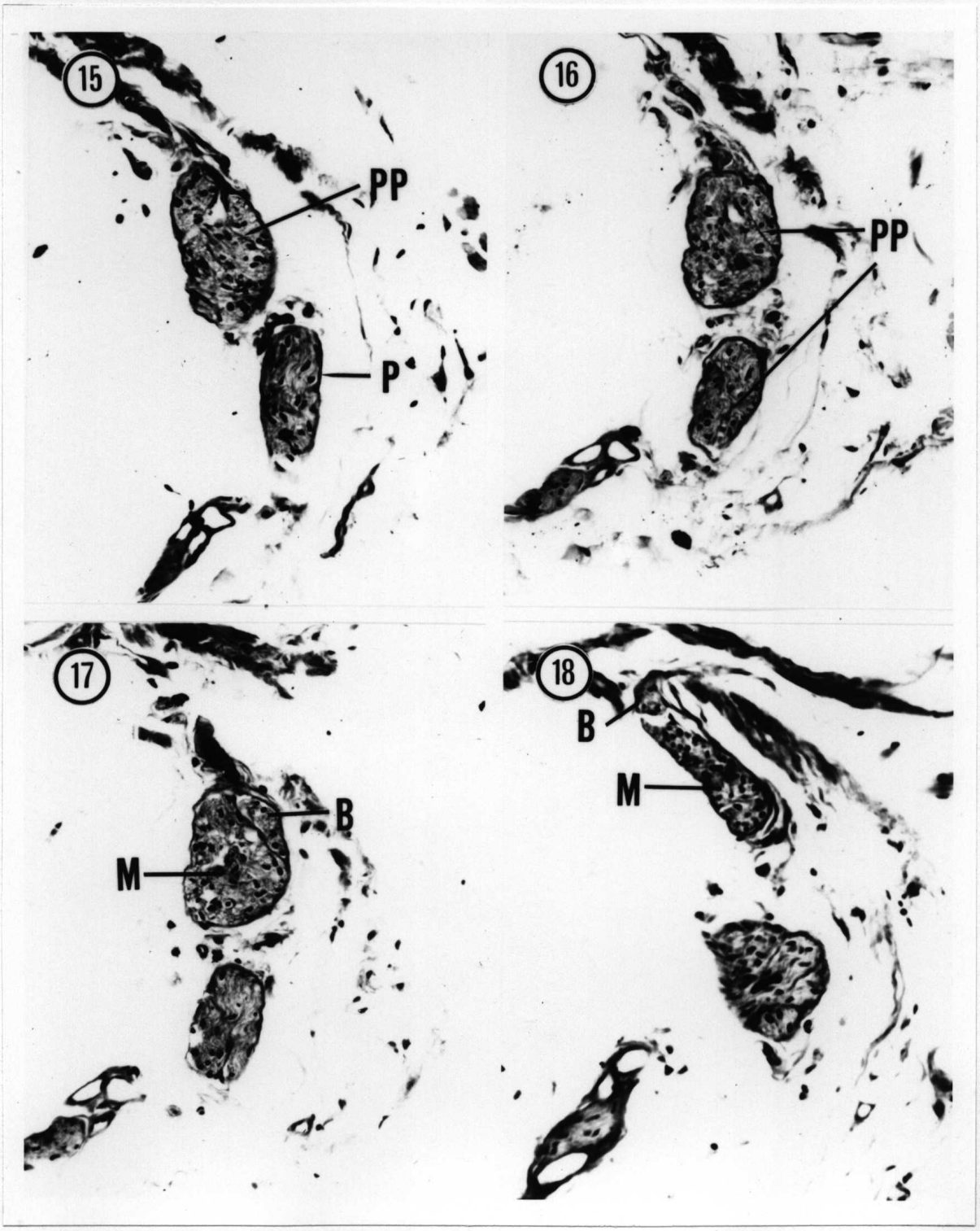


PLATE IX

Figure 19. (Detail of figure 16). Incomplete perineural partitions. The perineural partition of the upper nerve is almost three-fourths complete (arrows). The partition of the lower nerve is distinct in only two places (arrows). PAS and cresyl violet. 600X.

Figure 20. (Detail of figure 17). Complete perineural partitions. The complete perineural partitions of both nerves are bracketed by arrows. PAS and cresyl violet. 600X.

PLATE IX

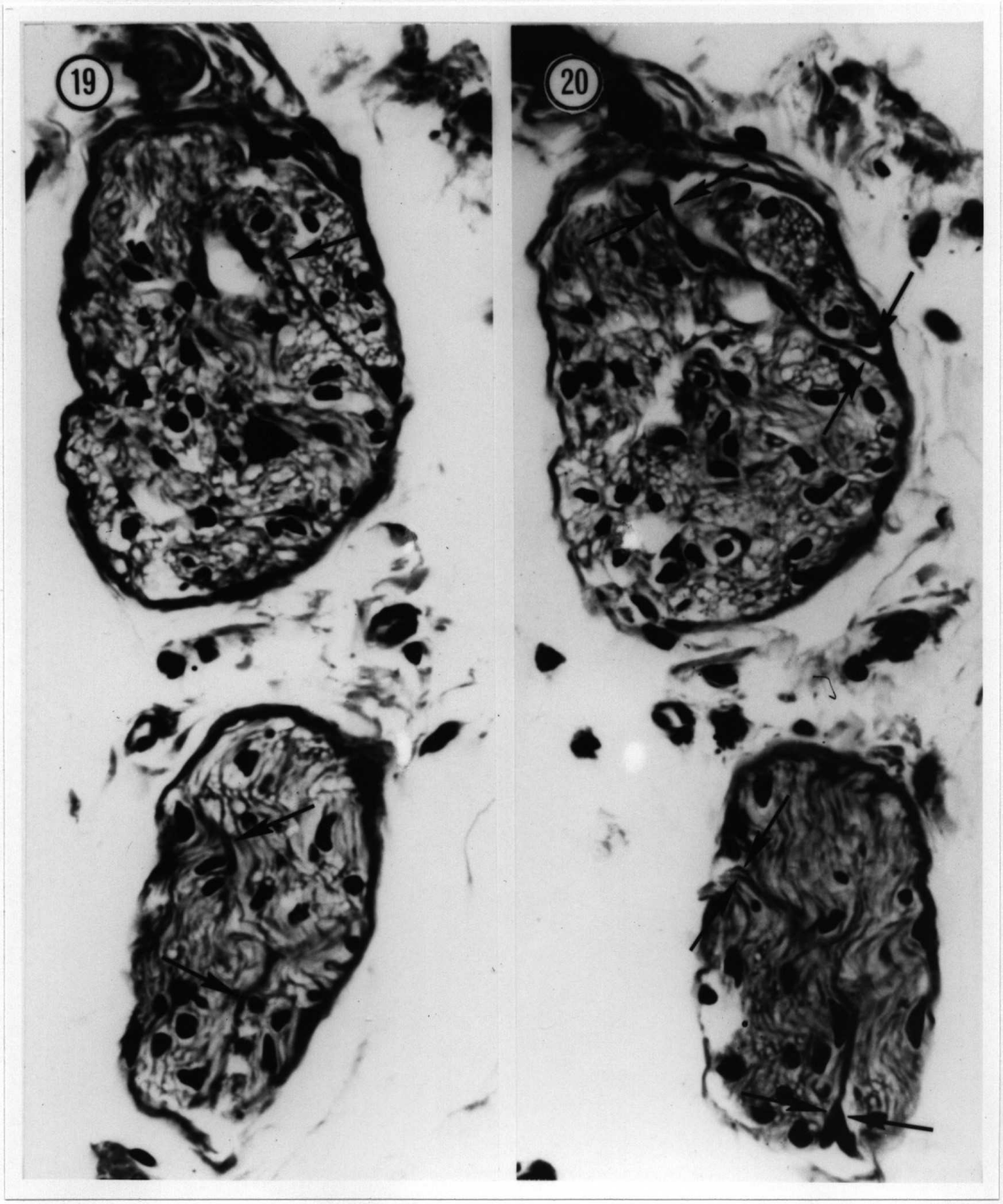


PLATE X

Figures 21-24. Blood vessel traverses perineurium; perineurium versus epineurium and endoneurium. This series of figures indicates that blood vessels enter a nerve by traveling longitudinally in the perineurium for some distance. In figures 22 and 23 the shape of the vessel has changed and in figure 24 the vessel is inside. Figures 21-23 clearly show that at least one layer of perineurium passes around each side of the vessel. The perineurium (P) with its nuclei (PN) is easily distinguished from the epineurium (EP) composed of collagen and reticular fibers with intermingled fibroblasts (F). Lightly stained endoneural collagen bundles are directly beneath the perineurium (arrows).

All figures are stained with hematoxylin (Harris's) and eosin. 600X.

PLATE X

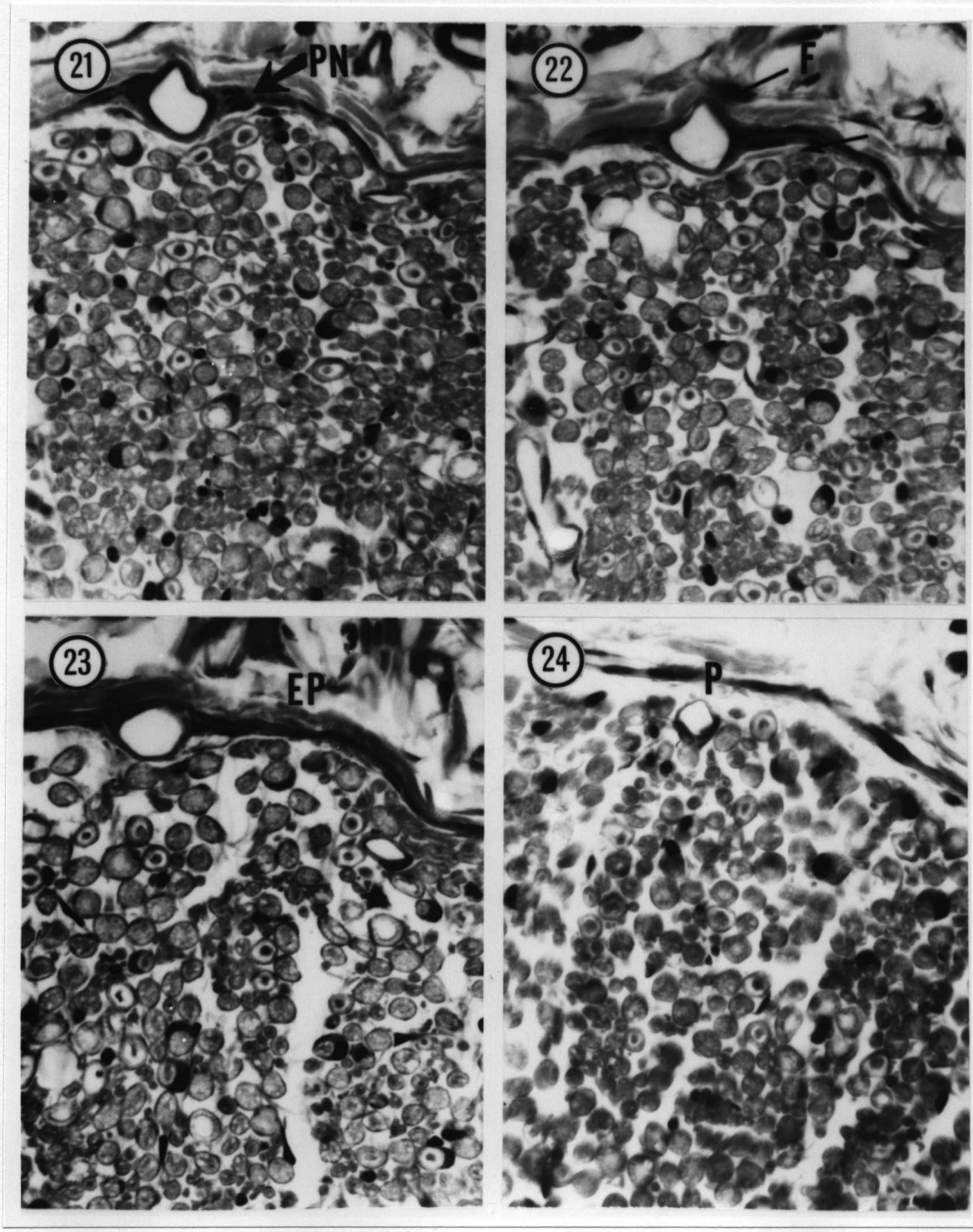


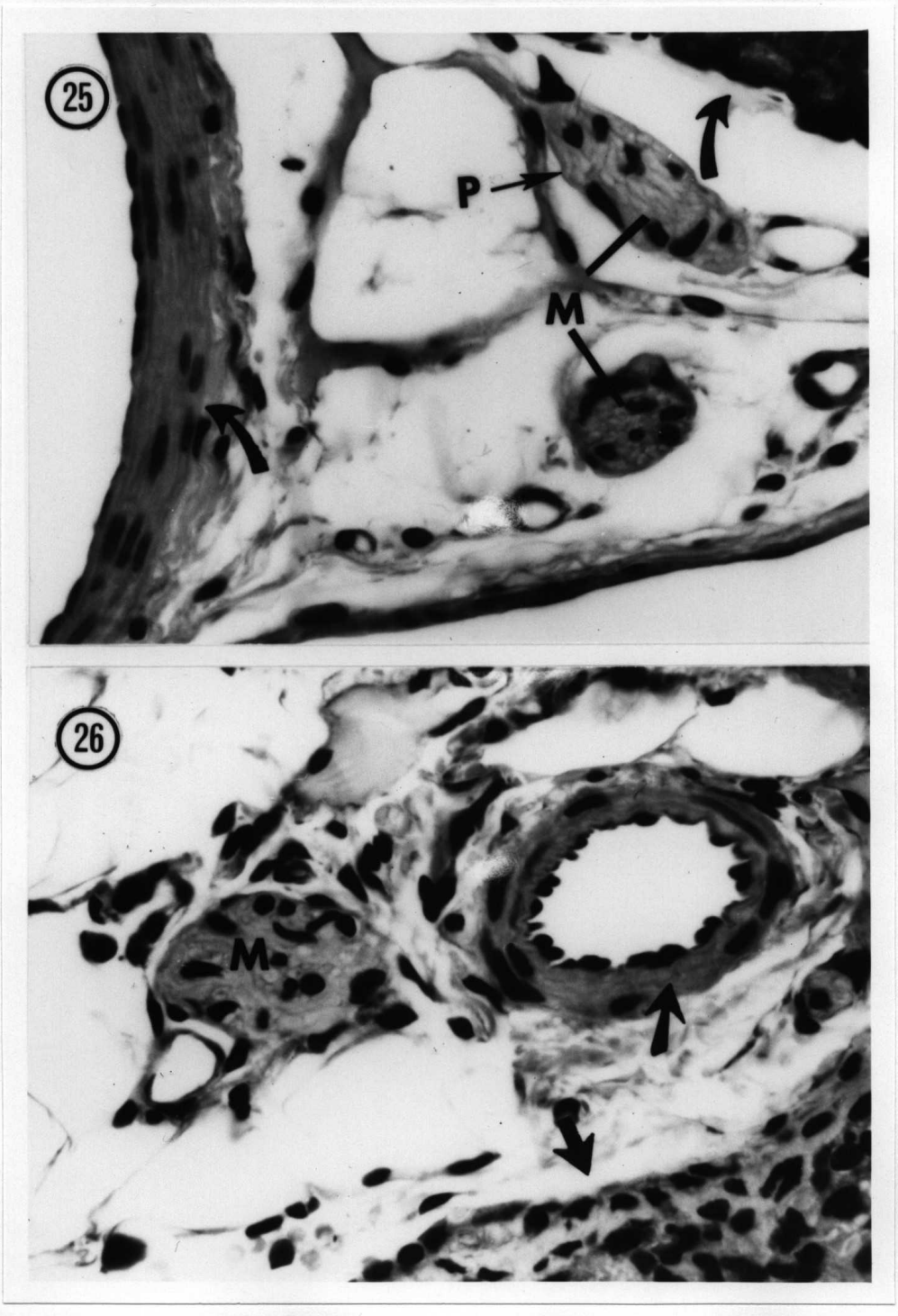
PLATE XI

Nerves associated with pancreas and spleen.

Figure 25. Two small compact nerves (M) are located in the interlobular connective tissue of the pancreas. Pancreatic tissue is at the upper right (arrow) and a patent artery at the left (arrow). The perineurium (P) is somewhat indistinct but can be seen. Hematoxylin and eosin. 600X.

Figure 26. A small compact nerve (M) in the hilus of the spleen (lower arrow) is associated with a partially collapsed arteriole (upper arrow). Hematoxylin and eosin. 600X.

PLATE XI



APPENDIX II

A TECHNIQUE FOR THE LIGHT MICROSCOPY OF TISSUES FIXED FOR FINE STRUCTURE

by

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INTRODUCTION

Biological fine structure is well preserved by buffered aldehyde fixation, a fact well known to electron microscopists. Also well known is the ease with which aldehyde-fixed tissue may be stained for light microscopy. These circumstances suggested that material known to be fixed adequately for electron microscopy might be prepared for light microscopy with profit (14). Accordingly, experimental animals were perfused with buffered aldehyde solutions. Following this special fixation, tissues were processed for light microscopy with the least possible alteration of standard techniques. This paper presents a full report of the outcome of these experiments.

PROCEDURE

Fixation:

Young adult virgin female rats of the Sprague-Dawley strain were perfused by the method of Palay et al. (11). Either phosphate buffered glutaraldehyde or formaldehyde (15), or a cacodylate buffered paraformaldehyde-glutaraldehyde mixture (5) was substituted for OsO_4. The height of the aortic perfusion column was reduced from five to three feet to prevent capillary rupture in unprotected soft tissues. When glutaraldehyde or formaldehyde was used alone, 10-20 ml of balanced salt solution were permitted to flow through a cannula as it was being inserted into the ascending aorta. Glutaraldehyde perfusion (200-250 ml) was terminated after 20 minutes, but the formaldehyde (500-700 ml) was permitted to flow at a reduced rate for three hours. Both techniques were followed by balanced salt solution for 20-30 minutes. When using Karnovsky's fluid, 10-20 ml of washout solution (isotonic fixer) preceded the perfusate (200-250 ml),

which was permitted to flow in full strength for 15-20 minutes. Optionally, perfusion was followed by a 10-15 minute rinse with the washout solution. The entire procedure was carried out at room temperature (4). Usually the perfusion was generally distributed but selected areas could be emphasized by means of arterial clamps. For purposes of comparison, some fresh tissues were fixed by overnight immersion in unbuffered 10% formalin.

Excision:

The extreme brittleness of tissues fixed by aldehyde perfusion called for special precautions. A minimum of handling, bending or twisting was essential to prevent detrimental cracking of the tissue mass. Unprotected soft structures suffered the least damage if removed with surrounding tissues. For example, undamaged peripheral nerve required concurrent removal of adjacent tissues, intact and without mechanical disturbance. Tissues surrounded by skeletal elements such as the central nervous system, orbital contents, etc. were obtainable only after careful removal of the bony encasements. During subsequent cutting, brisk chopping strokes with fresh blades were necessary to avoid compression and consequent damage. The cutting itself was always performed with the blocks immersed in the appropriate buffer solution, which could also serve for temporary storage.

Dehydration:

Dehydration was begun by adding the first ethanol solution directly to the buffer in which the tissue was immersed. Ascending concentrations of ethanol (30, 50, 70, 80, 95%, absolute) were added slowly to prevent damage by turbulence. Drop-by-drop addition was expedient with the lower concentrations. Pipette control of all fluid changes was found to be pre-

ferable to decanting. Damage by surface tension was avoided in the handling of especially delicate tissues (leptomeninges, etc.) by maintaining complete immersion of the blocks in fluid throughout all procedures.

Embedment:

Most tissues obtained by this procedure were unusually hard and required paraffin of high melting point (60-62°C) or double embedments containing celloidin (17). The matching hardness of these embedding media also helped prevent separation of tissue elements when the sections were heated and water-spread during mounting. However, softer tissues such as kidney could be embedded in 55°C paraffin and could be cut without compression. At the risk of less effective clearing, chloroform was substituted for xylene whenever the latter seemed to produce unmanageable hardness.

Microtomy:

Certain modifications of standard microtomy techniques were required because of the hardness noted above. Steel knives were hand sharpened on lightly etched glass plates by the method of Hillier (1), a technique originally developed for ultrathin sectioning. It was essential to fit the knives with single rear supports for accurate three-point suspension, thus assuring uniform bevels. Freshly sharpened knives were examined by the scattered light technique (13). An edge was considered satisfactory when examination at 100X revealed a fine bright line without a "necklace" pattern. A paraffinized cardboard pill box cut to fit the knife was used for fluid reception of sections. Distilled water and 15% acetone were the fluids of choice. Sections 4μ thick or less could be obtained routinely without interference by humidity or static electricity. Transfer of individual sections to glass slides was done by means of small loops

or forceps. Subsequent mounting procedures followed standard methods. When a dry knife was used, sections thinner than 7-8μ were difficult to obtain and were significantly affected by atmospheric conditions.

Staining:

Standard techniques for light microscopy (3, 7, and others) yielded adequate results. However, there was a general tendency to overstain and background color interfered with differentiation. These difficulties were corrected by altering concentrations and exposure times. Aldehyde blocking agents were used in a few cases. One dye substitution indicated below was made due to staining failure. Although chromatic values differed slightly from those of more familiar preparations, the colors were in general well saturated. The results obtained with some commonly used methods are summarized below.

Hematoxylin and Eosin (Harris): Chromatic balance adequate after hematoxylin, ½ to 1 minute; eosin, 1 minute. Tendency of hematoxylin to override eosin thus corrected. A reliable general technique.

Hematoxylin (Heidenhain) and Picro-fuchsin: Hematoxylin destains readily after overnight immersion if controlled by visual inspection. Overstaining in picro-fuchsin avoided by brief (15-30 second) immersion and regulated differentiation. Histological detail sharp.

Weigert's Elastin: Positive for elastic tissue but differentiation masked by background color. Shorter staining times not helpful after glutaraldehyde fixation.

Nissl (cresyl echt violet): An excellent stain for cytological detail in most cell types. Nissl substance in nerve cells less conspicuous than usual after glutaraldehyde fixation.

Reticulin (16): Strongly positive with standard procedures. Picro-

fuchsin irregular and must be adjusted to individual tissue. Excellent for fine detail of reticular fibers.

Masson's Tri-chrome: Aniline blue substituted for light green because of failure of the latter to stain. Good differentiation between cellular and extracellular formed elements.

Mallory's Tri-chrome: Irregular and unpredictable, but useful in some tissues.

PAS and Allochrome: Good differentiation hard to obtain, especially after glutaraldehyde fixation. Blocking agents such as aniline chloride (6) or aniline in glacial acetic acid (8) help if used before periodic acid. Background color limits usefulness of these two techniques.

When attempting to standardize these procedures in another laboratory, it is well to bear in mind the local variability that characterizes chromatic staining techniques (i.e. pH of tap water differs from area to area).

DISCUSSION

The results obtained during this study show that the preparation of finely-fixed tissues for light microscopy is practicable, even though somewhat exacting. Blocks of tissues fixed by perfusion with buffered aldehydes differ from conventional preparations in three distinct ways. They are brittle, hard and their response to chromatic stains is somewhat different.

Brittleness is especially critical after perfusion, which fixes the tissues in situ prior to excision. Since tissues are customarily removed while soft and pliable, the operator should be aware of dangers not ordinarily encountered. Extreme care with all manual manipulations is the best

approach to this problem. The greatest danger occurs during tissue removal when slow precision and careful mechanical control are essential. After excision, however, brisk chopping strokes seem to lessen the damage caused by compression. Gentle handling in all subsequent steps prior to embedment is also a significant factor in preventing damage. The brittleness seems to be a general characteristic of these preparations and is not readily explainable. However, it is not surprising to encounter this phenomenon in material that has been rendered insoluble and consequently less flexible at the molecular level of organization. It may well be an unavoidable sequel to the quality of the fixation itself.

Hardness is also general among these tissues and likewise seems to be unavoidable. This is very likely due to the increased amount of formed elements preserved by the fixation. It becomes critical only during microtomy and may be offset by three separate devices; matching hardness in embedding media, specially sharpened knives, and fluid reception of sections. Forbidding difficulty in microtomy is encountered only with a few traditionally troublesome tissues, such as tendon in cross section, etc. Most organs and tissues may be cut successfully as described herein.

A peculiar difficulty was encountered during the mounting of paraffin sections. Certain tissues, such as those containing leptomeninges or peripheral nerve, tended to break up while the sections were being spread on the warming pan. Apparently, the kinetics of contraction and expansion in the embedding medium were too vigorous for the more delicate portions of the tissue, the brittleness of which has already been emphasized. This phenomenon could be prevented no matter where it occurred by adopting a celloidin-paraffin mixture for subsequent embedments.

The staining characteristics observed in this study are also attributable to the complete amount of tissue preserved. Absence of artifactitious spaces and lack of clumping among formed elements both imply a general homogeneity not present in conventional preparations. Moreover, it is reasonable to suppose that the affinity of chromatic stains for these fine structures would express itself as background color. This would apply even though the formed elements themselves were smaller than the resolution limit of the optical system used.

The usefulness of this technique to the research investigator hinges on recognition of certain limitations imposed by standard fixation techniques for light microscopy (2). Critical evaluation of these methods became available with the development of the electron microscope when techniques for plastic embedment (9) and ultramicrotomy (12) revealed conspicuous distortion and clumping of tissue elements after conventional fixation. The development of buffered fixing fluids (10) made possible the preservation of tissue fine structure by maintenance of constant pH during fixation. For more than a decade O_sO_4 was the fixer of choice and it followed that early studies in fine structure were based almost entirely on its use. However, accompanying verifications with the light microscope were discouraged by masking effects (due to the dark brown color of the tissue) and inhibition of subsequent chromatic staining. The more recent development of buffered aldehyde fixation (15) provided a new approach. Chromatic staining was no longer masked or inhibited and the quality of the fixation matched the best O_sO_4 preparations. An added stimulus was provided by refinement of perfusion techniques (11). The experiments herein reported have explored the practicability of this approach. It appears that biological tissues properly

fixed for fine structure may be subsequently prepared for light microscopy by a wide variety of standard histological techniques.

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